

**TYPE OF FERMENTATION AND THE BIOCHEMICAL
TAXONOMY OF THE GENUS CHLORELLA**

von

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INTRODUCTION

The literature on algal fermentation is rather scanty. Genevois (1927, 1928), whose reports appear to be the first about this subject, demonstrated that under anaerobic conditions Chlorella pyrenoidosa, Coelastrum proboscideum and Scenedesmus basiliensis produced carbon dioxide and a number of organic acids from various hexoses. He stated that algal fermentation in its mechanism resembles the one found in yeast and animal cells.

Barker (1935) found that under anaerobic conditions the colourless alga Prototheca zopfii produced quantitatively 2 moles of D(-) lactic acid for each mole of glucose consumed. He pointed out the similarity of this type of fermentation behaviour, i.e. quantitative conversion of 1 mole of glucose into 2 moles of lactic acid, to that of homolactic fermenting bacteria.

Gaffron (1939) showed that Scenedesmus and Chlorella pyrenoidosa produced a large number of organic acids by the processes of both endogenous fermentation and exogenous glucose fermentation.

Michels (1940) observed that Chlorella pyrenoidosa fermented glucose to lactic acid and other organic acids, whereas Scenedesmus produced mostly carbon dioxide and little organic acid. She noted that the unspecified organic acids of the Chlorella fermentation did not include acetic, pyruvic, fumaric or malic acids.

Gaffron and Rubin (1942) reported hydrogen, carbon dioxide and traces of lactic acid to be the products of the endogenous fermentation process of autotrophically grown Scenedesmus.

Reazin (1956) found a similarity between the Ochromonas malhamensis type of fermentation and a typical yeast type of alcoholic fermentation. Reazin (1956) and Reazin and Gibbs (1956) worked with heterotrophically grown Ochromonas malhamensis and gave the following stoichiometric relationship between glucose fermentation and the endproducts produced:

glucose $\longrightarrow$ $1.60 \text{ CO}_2 + 1.75 \text{ ethanol} + 0.18 \text{ lactic acid}$.

Damaschke (1957) mentioned that Chlorella pyrenoidosa, like Prototheca zopfii, converts 1 mole of glucose quantitatively into 2 moles of lactic acid. He also observed that glucose fermentation by Chlorella pyrenoidosa, after an initial period of acid production, showed hydrogen as a fermentation product after some days of anaerobiosis.

Warburg, Gewitz and Völker (1957) demonstrated the configuration of lactic acid produced by Chlorella to be D(-) lactic acid.

Weis and Mukerjee (1958) noted the following three patterns of acid production among species of Chlorella and other algae:

(1) Chlorella pyrenoidosa (strains Burk and Emerson), Ankistrodesmus braunii and Scenedesmus produced lactic acid as the sole major product.

(2) Chlorella sp. (strain Marburg), Chlorella miniata and Chlorella ellipsoidea produced little or no acid.

(3) Chlorella vulgaris produced acetic acid as the major acid product and in addition ethanol and carbon dioxide.

Gibbs (1962), in his review, mentioned three types of algal fermentation: purely lactic acid; lactic acid, ethanol and carbon dioxide; and acetic acid, carbon dioxide and ethanol. He stated that in addition to these products some algae also produce hydrogen.

Syrett and Wong (1963), working with Chlorella fusca var. vacuolata (= Chlorella pyrenoidosa), have attempted to account quantitatively for the endproducts of glucose fermentation. They found, in agreement with Weis and Mukerjee (1958), that both acetic acid and lactic acid were important products. But in addition they reported that a considerable quantity of formic acid was produced as well as small quantities of carbon dioxide and hydrogen.

Karlander and Krauss (1966) have also reported acetic acid and formic acid production by Chlorella vulgaris (Emerson strain).

Kessler et al. (1963) started an investigation of the biochemical taxonomy of algae. They demonstrated that Chlorella strains can be classified on the basis of physiological and biochemical characters such as the presence or absence of the enzyme hydrogenase, the production of secondary carotenoids under nitrogen-deficient conditions (Kessler et al., 1963; Kessler and Czygan, 1965; Kessler, 1967; Kessler et al., 1968; Kessler and

Zweier, 1971), the capability of liquefying gelatin (Soeder, 1963; Kessler and Czygan, 1965; Kessler et al., 1968), nitrate reduction (Kessler and Zweier, 1971), thiamine requirement (Kessler and Zweier, 1971), acid limit of growth and thermophily (Kessler, 1965, 1967, 1972; Kessler and Czygan, 1965; Kessler et al., 1968; Kessler and Zweier, 1971). With their recent work on the base composition of DNA (Hellmann, 1973; Hellmann and Kessler, 1974) and salt tolerance (Kessler, 1974), they found more evidence concerning the striking differences between the species in the genus Chlorella.

An attempt was made by Begum and Syrett (1970) to classify 10 strains of Chlorella based on the quantity of lactic acid produced per mole of glucose fermented anaerobically. These strains were grouped under 4 categories. They stated that their results agreed with the classification system based on other biochemical and physiological characters as proposed by Kessler et al. (1963) and Soeder (1963). They added that, as their results were limited, no generalization could be made.

The present work is an attempt to study the products of glucose fermentation by 87 Chlorella strains, and to relate them to the taxonomy of the genus.

MATERIALS AND METHODS

Organisms and Culture Conditions

The 87 Chlorella strains used in this investigation are the same as in the work of Hellmann and Kessler (1974) and Kessler (1974), with the exception of Chl. vulgaris f. tertia 211-18. Details about the source of the strains can be found in the work of Kessler (1967), Kessler et al. (1968) and Kessler and Zweier (1971).

The algae were grown in the culture media of Kessler and Czygan (1970) and Kessler and Zweier (1971). Chlorella homosphaera 211-8e (Cambr.) was grown in the normal culture medium of Kessler and Czygan (1970) with an addition of 0.2% yeast extract (Merck).

Cultures were grown for 7 days at 25°C with a light intensity of 6000 lux and were aerated with air containing 2.5% (v/v) CO₂. They were starved before use by aeration for a further 18 hr in darkness. The cells were harvested by centrifuging at 500 x g, washed 4 to 5 times with deionized water and resuspended in 2 mM MgSO₄ . 7H₂O. This was adjusted in such a way that each ml contained roughly 15 to 20 mg of dry weight of algae. Before the algal suspension was pipetted into manometric flasks, the algal samples were examined under the microscope for any possible contamination by bacteria.

Determination of Dry Weight

Crucibles (Schott G₃) were filled with asbestos suspension, and the water was suctioned off. They were left in a hot oven at 105°C for two hours, and then transferred to a desiccator. After cooling the weights of the crucibles were taken. 5 ml of algal suspension was pipetted into each crucible and washed with deionized water. The crucibles were again transferred to the hot oven, left for two hours at 105°C, and later cooled in the desiccator and weighed. Dry weight was expressed in mg per ml.

Manometry

Gas exchange was measured by Warburg manometry at 25°C in darkness. The experiments were performed under two different conditions. Each flask contained 3 ml cell suspension and 1.5 ml 0.01 M KHCO₃; 0.2 ml glucose solution (containing 4 μmoles) and 0.3 ml deionized water were filled separately in the two side arms. The experiments were performed in quadruplicate. Control flasks contained no glucose. The final volume in each flask was 5 ml.

In a second set, 6 flasks were taken, each containing 3 ml cell suspension in 0.033 M potassium phosphate buffer, pH 7.1. In flasks 1 and 2 no glucose was added and the volume was adjusted to 5 ml by filling a suitable amount of deionized water into one side arm. Flasks 3 and 4 contained, in addition to cell suspension and potassium phosphate buffer, 0.2 ml glucose solution in a side-arm and 0.2 ml KOH (10%) in the centre well with filter paper (assumed to be equal to 0.1 ml). Flasks 5 and 6 were similar to 3 and 4 except that the KOH was omitted and the volume was adjusted to 5 ml with deionized water.

The final volume in each of the flasks was 5 ml. Flasks containing bicarbonate were gassed with a mixture of 95% N_2 : 5% CO_2 (v/v) mixture. The flasks containing potassium phosphate buffer were gassed with oxygen-free pure nitrogen.

After an equilibration period of 10 min, glucose from the side arm was tipped into the cell suspension in the main compartment of the flask. Readings of flasks for gas exchange were taken for 5 hr after glucose was added. When fermentation was complete, the cell suspension from the flask was washed into a centrifuge tube. After centrifuging, the supernatants were made up to 10 ml from each flask and were stored at $-20^{\circ}C$. In all these experiments the samples were collected for the analysis of fermentation products from the flasks containing potassium bicarbonate.

Analysis of the fermentation products

Estimation of glucose

Glucose was determined with the anthrone method of Yemm and Willis (1956), with some modification in the following manner. 100 mg anthrone was dissolved in 50 ml conc. H_2SO_4 . (Conc. H_2SO_4 was used undiluted for the reason that with diluted H_2SO_4 the final solution after the test formed a precipitate which made the reading in the spectrophotometer impossible). The anthrone reagent was allowed to stay for 40 min or more with constant stirring and shaking. The reagent was well protected from any sort of contamination. Only 2 ml of the anthrone reagent were taken and 1 ml of test solution was added, and the further experimental procedure was carried out as mentioned by Yemm and Willis (1956). It gave good and reproducible results.

The anthrone reagent (2 ml) was pipetted into a thick-walled Pyrex tube and chilled in ice water. The sample solution (1 ml) was layered on the acid reagent, cooled for a further 5 min and then thoroughly mixed while still immersed in ice water. The tubes were loosely stoppered, heated for 10 min in a vigorously boiling constant level water bath and then cooled in ice water for 5 min. After cooling, extinction was read in a spectrophotometer at 620 nm using 1 cm cuvettes with water as a reference.

Deproteinization of the sample

Deproteinization was done after the method of Somogyi (Neish, 1952). 2 ml of zinc sulphate solution (25% w/v) was added to the fermentation solution and was titrated (using a pH meter) with 1N sodium hydroxide solution till the pH of the solution was 7.6 - 7.8. The solution volume was then adjusted as required with deionized water, centrifuged and the supernatant was stored at -20°C for further analysis.

Estimation of lactic acid

Lactic acid was estimated by the method of Hullin and Noble (1953) as modified by Nanni and Baldini (1964) (see Dawes et al. 1971). 1 ml of protein-free solution was pipetted into a centrifuge tube which was graduated exactly at 10 ml. One ml 20% copper sulphate was added, and the mixture was made up to a final volume of 10 ml with deionized water. After addition of 1 g $\text{Ca}(\text{OH})_2$ the tube was stoppered and shaken vigorously for 30 sec periods at intervals of 5 min for 30 min. The stopper was then removed and the tube centrifuged.

One ml of supernatant solution was pipetted into a stoppered Pyrex tube. The bottom of the tube was placed in an ice-bath and 0.05 ml 15% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was added, followed by 6 ml conc. H_2SO_4 , which was added dropwise from a burette with continuous shaking. When all the acid was added, the tube was vigorously shaken. The stoppered tube was transferred to a water bath ($60 \pm 1^{\circ}\text{C}$) for 30 min, then removed and cooled to $8 - 10^{\circ}\text{C}$. 0.1 ml of alcoholic p-hydroxydiphenyl was added and mixed by vigorously shaking (p-hydroxydiphenyl solution: 150 mg p-hydroxydiphenyl was dissolved in 10 ml absolute ethanol). The tube was then incubated in a water bath at $29 \pm 1^{\circ}\text{C}$ for 30 min, followed by 90 sec in a boiling water bath, a treatment which removes excess p-hydroxydiphenyl. Immediately after this treatment the tube was cooled in the ice water and the extinction at 570 nm determined against a blank prepared by taking water through the whole procedure.

Samples containing more than 120 μg lactic acid were diluted with conc. H_2SO_4 / H_2O (6 : 1 v/v) prior to reading the extinction.

Enzymatic test for D(-) lactic acid

D(-) lactic acid present in the samples was identified by the method described by Gawehn and Bergmeyer (1970), which is based on the reaction



The NADH produced is measured spectrophotometrically at 340 nm. It is proportional to the D(-) lactate concentration.

Relatively high concentrations of NAD and D(-) LDH are necessary for a sufficiently fast reaction. In the present investigation attention was given to identify the presence of D(-) lactic acid.

All the solutions required for the experiment were prepared freshly. The enzyme D(-) lactic dehydrogenase (D-LDH) (D-lactate: NAD oxidoreductase, EC 1.1.1.28) used was from Lactobacillus leichmannii (supplied by Boehringer Mannheim).

3 ml glycine buffer were pipetted into the cuvette, 0.2 ml NAD solution and 0.1 ml deproteinized test solution were added and mixed thoroughly. Extinction E_1 was read at 340 nm in a spectrophotometer. Cuvettes used were of 1 cm thickness. 0.01 ml suspension of D-LDH was added and mixed thoroughly. Extinction was followed for 60 min, and a final reading E_2 was done. The presence of lactic acid was determined by calculating the difference $E_2 - E_1 = E$.

Throughout the experimental procedure water was used in place of test solution and taken as a blank.

The above test has been conducted only to demonstrate D(-) lactic acid present in the sample, and attention was not given to quantitative results.

Identification of ethanol

Determination of ethanol was done by the method of Bernt and Gutmann (1970). With this method, which is relatively specific for ethanol, there are interferences from other alcohols, especially n-propanol and n-butanol. These may be overcome by the very different rates of oxidation of the interfering alcohols. Ethanol is determined using alcohol dehydrogenase, the

production of NADH being followed spectrophotometrically:



The test cuvette contained pyrophosphate-glycine-semicarbazide buffer (3.0 ml), NAD solution (0.01 ml) and the sample (0.04 ml). The contents of the cuvettes were thoroughly mixed, and E_1 was determined at 340 nm using 1 cm cuvettes with water as a blank reagent.

Alcohol dehydrogenase (0.01 ml) was mixed into the cuvette, and this was allowed to stand for 70 min at 25°C. E_2 was then determined. Standard ethanol solution was also used along with the test solutions. In the present investigation qualitative identification of ethanol rather than quantitative determination was attempted.

The presence of alcohols was also determined confirmatively using gas chromatography. Details about this technique are given separately.

Glycerol

Glycerol was determined by the method of Lambert and Neish (1950) in which formaldehyde formed on periodate oxidation is measured.

2 ml test solution were pipetted into a Pyrex test tube and 10 N H_2SO_4 (0.1 ml) was added, followed by 0.5 ml periodate reagent. After 5 min 0.5 ml 1 M sodium arsenite was added and mixed thoroughly. After 15 min the volume was made up to 10 ml with deionized water and 1 ml was removed for estimation.

A 1 ml sample was pipetted into a Pyrex test tube, 10 ml chromotropic acid reagent was added, and the test tube was then heated on a boiling water bath for 30 min in diffuse light. The tube was cooled to room temperature and read in the spectrophotometer at 570 nm using 1 cm cuvettes. A blank and a set of standards were also run with each set of determinations.

Identification of organic acids by thin layer chromatography

Formic, lactic, acetic and succinic acids were identified as the nonvolatile sodium salts by thin layer chromatography (TLC) directly from the alkaline supernatant liquid without distillation or concentration and as the steam distillate prepared by the method of Friedemann (1938), and also by the method of White and Starr (1971).

For the preparation of thin layer plates the method given by White and Starr (1971) was applied or the ready-made plates supplied by Merck were used.

The plates were developed in the Kontes sandwich chamber using 50 ml solvent at a depth of 1 cm. The solvent mixture used was absolute ethanol-concentrated ammonium hydroxide-deionized water in proportions of 80 + 4 + 16, prepared immediately before use.

After a development period of 60 min, the plate was dried briefly with a hair dryer and sprayed with a 0.02% alcoholic solution of methyl red. The plate was dried in the 100°C oven for 20 min to evaporate the ammonia and thus develop the red colour of the acid spots.

Volatile acids

The volatile acids were determined after steam-distillation (Friedemann, 1938), as described by Syrett and Wong (1963) in the following manner. 10 ml samples of cell suspension were added to 1 ml 10 N H_2SO_4 , and the mixture was steam-distilled after the addition of 3 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3 ml 20% (w/v) sodium tungstate and 0.5 ml 40% (w/v) KOH. The distillate of 125 ml was collected. These distillates usually contain variable quantities of CO_2 which was removed before titration. For this purpose the 125 ml of distillate was transferred to a modified Erlenmeyer flask and was vigorously titrated with freshly prepared 0.01 N NaOH, with phenol red as indicator. After titration the distillate was made alkaline with 1 N NaOH (freshly prepared every time), concentrated to 30 ml in vacuum at low temperature, using a Rotary evaporator. This 30 ml solution was redistilled from a flask containing 12 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g HgO and 0.5 ml 10 N H_2SO_4 . This second distillation removes any formic acid, lactic acid and (or) pyruvic acid that may have been present in the

steam distillate. The recovery of known amounts of acetic acid after both distillations was about 95% (Syrett and Wong 1963).

Formic acid

The neutralized steam distillate (from the above procedure) was made alkaline and blown to dryness with cool air, and formic acid was determined by the method of Perlin (1954). In this method the formic acid was oxidized to carbon dioxide by lead tetra-acetate; the gas produced was measured manometrically in the following manner. The dried steam distillate was dissolved in 5 ml 90% (v/v) acetic acid. 2 ml of the sample solution were placed in a Warburg flask; the side arm of the flask contained 0.5 ml of freshly prepared reagent (20 mg potassium acetate and 25 mg lead tetra-acetate per ml in 90% (v/v) acetic acid). The reaction was carried out at 30°C and was complete in 2 hr. Standard sodium formate solutions were treated in the same way as the steam distillates at the same time.

Analysis of volatile reducing substances and volatile acids by gas chromatography

Selection and preparation of the glass column for gas chromatographic analysis of the samples was done as suggested by Kaiser (1969).

Extraction of alcohols from the samples was done by the method of Hugo et al. (1972) with a slight modification. Hugo et al. suggested ether extraction, but in the present work benzene was used. Deproteinized samples from Warburg experiments were saturated with $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and brought to a pH of 9-10 with NaOH. Then they were extracted 20 times with 1 ml benzene. The combined fractions were concentrated to a volume of 0.6 ml with a stream of nitrogen at 0°C. After removal of traces of water by solid sodium sulfate, the samples were analyzed with a flame ionization detector-equipped gas chromatograph. Standard solutions prepared with water and benzene were run first. The advantage with the selected glass column is that it tolerates the presence of water in the samples. The standard solution contained ethanol, isopropanol, butanol, acetic acid and butyric acid. Salts like pyruvate, succinate, citrate, lactate and acetate were also tried by dissolving them in water as well as in benzene.

The peaks obtained from the samples were compared to the standard solution peaks, and the substances were analyzed. Some unidentified peaks are termed "unknown peaks".

Equipment and conditions used: Carlo Erba Model 2400 V gas chromatograph; Fractovap N^o 2400; carrier gas nitrogen (N₂); pressure 1.3 kg/cm²; flow 25 ml/min; auxiliary gas air-pressure 1.5 kg/cm²; hydrogen pressure 0.6 kg/cm²; temperature programme column: 80-250°C; Vap. 200°C; temp 5°/min. Column length 2 meters; diameter 3 mm; Chromosorb 102; glass column. All glass system. Detector background signal-scale 100. Attenuation: input 100; output-var. Recorder: 2.5 mvf.s. chart 5 mm/min.

In the present work both programme and isotherm (temp. 200 and 250°C) were applied for both alcohols and volatile acids. The samples were not quantitatively analyzed, and attention was given to qualitative identification.

RESULTS

Chlorella fusca var. vacuolata Shihira et Krauss

All the 10 strains of this taxon showed the same behaviour in producing lactic acid, acetic acid, formic acid, ethanol, carbon dioxide and hydrogen as their glucose fermentation products. Taking into consideration the quantity of lactic acid, they behaved in a similar way by producing 0.7 to 0.9 mole of lactic acid per mole of glucose fermented. The test done with the enzyme D(-) lactic dehydrogenase gave a positive reaction showing that the lactic acid produced was D(-) lactic acid. These strains did not produce glycerol. Ethanol and acetic acid have been confirmed gas chromatographically. Hydrogen production was noticed in the manometric readings of the flasks containing algal suspensions in potassium phosphate buffer (with KOH in the centre well).

Chlorella fusca var. fusca Shihira et Krauss

The only strain of this taxon behaved similarly to the group of Chlorella fusca var. vacuolata in producing 0.7 mole of lactic acid per mole of glucose fermented and, in addition, formic acid, ethanol, carbon dioxide and hydrogen. The presence of acetic acid, though indicated gas chromatographically as well as by thin layer chromatography, could not be proved unequivocally, whereas it has been clearly shown for the strains of Chlorella fusca var. vacuolata when the steam distillate was directly injected into the gas chromatograph. Otherwise this strain does not seem to differ from the other group. Glycerol was not produced. A test for 2,3-butanediol was not carried out.

Chlorella fusca var. rubescens

(Dangeard) Kessler et al.

The only strain available, which is of marine origin, differs from the other two closely related taxa Chlorella fusca var. vacuolata and Chlorella fusca var. fusca in producing about 1.7 mole of lactic acid per mole of glucose fermented and also trace amounts of glycerol. Other products identified are formic acid, carbon dioxide and hydrogen. Ethanol was gas chromatographically confirmed.

Table 1

Fermentation products from glucose produced by different strains of the genus *Chlorella* under anaerobic conditions.

Species	Strain	Lactic acid	Acetic acid	Formic acid	Ethanol	Propanol	Glycerol	Carbon dioxide	Hydrogen	2,3-butanediol not identified	2,3-butanediol
<u>Chl. fusca</u>	211-8a	+	+	+	+	-	-	+	+		
<u>var. vacuolata</u>	211-8b	+	+	+	+	-	-	+	+		
Shihira et Krauss	211-8c	+	+	+	+	-	-	+	+		
	211-8e (Gött.)	+	+	+	+	-	-	+	+		
	211-8g	+	+	+	+	-	-	+	+		
	211-8h	+	+	+	+	-	-	+	+		
	211-11m	+	+	+	+	-	-	+	+		
	211-11n	+	+	+	+	-	-	+	+		
	211-15	+	+	+	+	-	-	+	+		
	C-1.1.10	+	+	+	+	-	-	+	+		
<u>Chl. fusca</u>	343	+	+	+	+	-	-	+	+		
<u>var. fusca</u>											
Shihira et Krauss											
<u>Chl. fusca</u>	232/1	+	-	+	+	-	+	+	+		
<u>var. rubescens</u>											
(Dangeard)											
Kessler et al.											

Table 1 -continued

<u>Chl.</u> <u>homosphaera</u> Skuja	211-8e (Cambr.)	Did not ferment glucose	+
<u>Chl.</u> <u>luteoviridis</u> Chodat	211-2a	-	-
	211-2b	-	+
	211-3	-	+
	211-4	-	+
	211-5a	-	+
	211-5b	-	+
<u>Chl.</u> <u>saccharophila</u> (Krüger) Nadson	211-1b	-	+
	211-1c	-	+
	211-1d	-	+
	211-1f	-	+
	211-9a	-	+
	211-9b	-	+
	211-1a	+	+
	C-1.3.1	+	+
<u>Chl.</u> <u>zofingiensis</u> Dönz	211-14a	-	+
	211-14b	-	+
	211-14c	-	+
	C-1.2.1	+	+

2,3 butanediol not identified

Table 2

Quantitative estimation of lactic acid per mole of glucose fermented. Results are given for those strains which produced lactic acid. Results for Chlorella protothecoides are not given (see under results).

Species	Strain	Glucose fermented (μ moles)	CO ₂ released from KHCO ₃ x moles/mole glucose fermented	Lactic acid moles/mole glucose fermented
<u>Chl. fusca</u> <u>var. vacuolata</u> Shihira et Krauss	211-8a	3.2	2.4	0.8
	211-8b	3.2	2.5	0.8
	211-8c	3.2	2.3	0.9
	211-8e(Gött.)	4.0	2.0	0.7
	211-8g	3.2	2.1	0.7
	211-8h	3.0	2.2	0.8
	211-11m	3.2	2.5	0.8
	211-11n	3.2	2.5	0.8
	211-15	3.2	2.3	0.7
	C-1.1.10	3.0	2.0	0.8
<u>Chl. fusca</u> <u>var. fusca</u> Shihira et Krauss	343	3.2	1.7	0.7

Table 2-continued

<u>Chl. fusca</u> var.				
<u>rubescens</u>				
(Dangeard)				
Kessler et al.				
	232/1	3.6	1.6	1.7
<u>Chl. saccharophila</u>				
(Krüger)	211-1a	1.0	1.7	2
Nadson	C-1.3.1	1.23	3.0	2
<u>Chl. zofingiensis</u>				
Dönz	211-14a	2.8	3.2	2
	211-14b	3.0	3.5	2
	211-14c	2.6	3.7	2
	C-1.2.1	3.1	1.1	1.4
<u>Chl. vulgaris</u> var.				
<u>vulgaris</u>	211-1e	3.2	1.06	1.2
Beijerinck	211-81	1.9	1.5	1.3
	211-8m	3.1	1.5	1.2
	211-11b	3.2	1.5	1.3
	211-11c	2.9	1.5	1.2
	211-11f	2.7	2.2	1.3
	211-11j	2.4	1.7	1.3
	211-11l	3.1	1.4	1.3
	211-11p	3.1	1.5	1.3
	211-11q	3.1	1.5	1.3

Table 2-continued

211-11s	1.3	2.2	1.5
211-11t	3.4	1.05	1.3
211-12	3.1	1.6	1.4
211-19	3.1	1.6	1.4
211-21	3.1	1.6	1.4
395	2.5	0.7	1.3
396	3.1	2.0	1.3
211-11r	2.8	1.8	2
211-30	Did not ferment glucose		
211-8k	3.0	2.3	1.5
211-11d	3.6	1.8	-
211-11k	2.5	1.8	2
211-32	3.0	2.0	1.3
211-33	3.0	2.0	1.3
211-34	2.5	2.0	1.8
C-1.1.8	3.6	0.8	1
C-1.6.7	3.2	1.0	1.3
Sless 1	3.5	1.0	2
Sless 2	3.5	1.0	2
211-31	2.5	2.9	2
1-9-30	3.6	2.9	1.3
Prag A14	3.6	0.5	2
211-4Oa	3.6	1	2
211-4Oc	3.5	1	2

Chl. vulgaris f.
tertia
 Fott et
 Nováková

Chlorella homosphaera Skuja

The only strain of this species (211-8e from Cambridge) did not ferment glucose under our experimental conditions. The only product that could be observed was hydrogen.

Chlorella luteoviridis Chodat

This group of 6 strains differs from the other species in producing glycerol as a main fermentative product. All the strains tested for glycerol by the method of Lambert and Neish (1950) gave the highest results as compared to other taxa which also produced glycerol.

When tested for ethanol enzymatically, this species gave the highest values and reduced the added NAD at a fast rate compared to the other groups which also produced ethanol. Though this enzymatic test is relatively specific for ethanol, it was suspected that glycerol or some other unknown product could have interfered. Gas chromatographic analysis proved to give variable results depending on the extraction methods employed. Thus these results were assumed to be doubtful until the presence of alcohol dehydrogenase can be confirmed in these strains.

No strain in this group produced lactic acid. Carbon dioxide was the other product identified.

Chlorella kessleri Fott et Nováková

The 7 strains of this species showed the same fermentative pattern in not producing lactic acid. In this respect Chlorella kessleri differs from other hydrogenase-containing taxa like Chlorella fusca var. vacuolata, Chlorella fusca var. fusca, Chlorella fusca var. rubescens and Chlorella vulgaris f. tertia.

All strains showed a very slow rate of fermentation. Glucose determination done after the experiment showed that most of the glucose remained without being utilized. The only strain which showed active gas production was strain C-1.1.12.

Ethanol could not be detected gas chromatographically. Enzymatic tests done with the enzyme alcohol dehydrogenase and NAD showed a slow reduction of NAD. Gas chromatographic analysis showed the presence of isopropanol as two small peaks arising in the temperature range of 130-140°C. By repeating the analysis with different extraction methods these two small peaks also

could be obtained. After consulting the standard curves, it was concluded to be isopropanol.

Other products identified are formic acid, carbon dioxide and hydrogen. Based on the character of not producing lactic acid and producing iso-propanol, this group can be delineated from other taxa producing ethanol and lactic acid.

Chlorella saccharophila (Krüger) Nadson

Of the available 8 strains in this group, two strains (211-1a and C-1.3.1) differ from the other ones in producing lactic acid as a fermentative product. It is interesting to note that these two strains were shown by Hellmann and Kessler (1974) to have a base composition of DNA different from that of the other strains. The other strains, though producing glycerol in common with the strains 211-1a and C-1.3.1, did not produce lactic acid. Carbon dioxide was the other product identified. Besides strains 211-1a and C-1.3.1, strain 211-9b showed higher carbon dioxide liberation than the other ones. The strains which did not produce lactic acid in this group showed a character similar to Chlorella luteoviridis in fermenting negligible amounts of glucose. Strains 211-9b and 211-1f exhibited high values when tested for glycerol.

Chlorella zofingiensis Dönn

All the 4 strains of this taxon produced lactic acid both by endogenous fermentation and by fermentation of added glucose. In addition, carbon dioxide was identified. Strain C-1.2.1, like the strains of Chlorella vulgaris var. vulgaris, produced 1.4 moles of lactic acid per mole of glucose fermented, whereas the other 3 strains showed a quantitative conversion of glucose into lactic acid. Glycerol was not produced, and ethanol was not identified.

Chlorella minutissima Fott et Nováková

The only strain fermented considerable amounts of glucose but did not produce lactic acid. Glycerol was found to be produced in traces. In addition, carbon dioxide was formed.

Chlorella vulgaris var. vulgaris Beijerinck

Except for the strains 211-11r and 211-30, which also proved to be different in the work of Hellmann and Kessler (1974) and Kessler (1974), the other 17 strains of this taxon showed the same fermentative behaviour in producing 1.3 to 1.5 moles of lactic acid per mole of glucose fermented. Strain 211-11r differed from the others in converting glucose quantitatively into lactic acid. 211-30 did not ferment glucose. Other products identified in this group were carbon dioxide and glycerol. Those strains which did not show glycerol when analyzed gas chromatographically showed traces of ethanol. Since these results could not be repeated in a reproducible way, they have not been included in the table.

Chlorella vulgaris f. tertia Fott et Nováková

Regarding the quantity of lactic acid produced per mole of glucose fermented, the group of strains included under Chlorella vulgaris f. tertia varied considerably from uniformity (cf. also Hellmann and Kessler, 1974). Strain 211-11d did not produce lactic acid. This is the only strain which did not respond to both the chemical and the enzymatic tests for lactic acid. All the other strains in the group responded to the test done with D(-) lactic dehydrogenase. Strain 211-11d fermented 3.6 μ moles of glucose out of 4 μ moles that had been added. Carbon dioxide production was also high. Other products identified were hydrogen and formic acid.

Strains Prag A 14, 211-40a, 211-40c, 211-31, 211-34, 211-11k, Sless 1 and Sless 2 produced in general high quantities of lactic acid and only traces of formic acid. Other products identified for these strains were carbon dioxide and hydrogen. Especially the strains Sless 1 and Prag A 14 produced such high quantities of lactic acid that it exceeded the amount of glucose from which it could be accounted for. The same strains showed variable results for formic acid. In both cases, the endogenous rate was found to be very high.

Strains 211-8k, 211-32, 211-33, C-1.1.8, C-1.6.7 and C-1.9.30 produced 1.4 moles of lactic acid per mole of glucose fermented. This pattern of fermentation closely resembles that found with Chlorella vulgaris var. vulgaris.

Chlorella protothecoides Krüger

The 14 strains of this species showed a uniform fermentative behaviour in producing lactic acid, glycerol and carbon dioxide. In general they fermented very small amounts of glucose. After the experiment most of the glucose remained unutilized. Tests done for lactic acid proved that these strains produced it. Enzymatic tests done with D(-) lactic dehydrogenase gave positive results indicating that the lactic acid produced is D(-) lactic acid. Quantitative results for lactic acid are not given, since only very small amounts of glucose disappeared and the lactic acid produced was more than could be accounted for. Probably lactic acid has been also produced endogenously.

DISCUSSION

The results given in table 1 show that the Chlorella species can be distinguished on the basis of their patterns of fermentation. The results agree with the classification system proposed by Kessler (1967, 1974) and also with the results later shown by others to agree with this system (Fott and Nováková, 1969; DaSilva and Gyllenberg, 1972).

It may not be proper to claim that the present investigation has detected all the fermentation products produced from glucose under the conditions specified. There might exist many other products in such minute amounts which might have gone undetected or could not be identified with the methods employed.

Syrett and Wong (1963) worked with Chlorella fusca var. vacuolata on products of glucose fermentation and reported that 15% of the added glucose were incorporated into polysaccharide, and they attributed the discrepancy in the carbon balance sheet in their experiments to other synthetic reactions and suggested that other unidentified fermentation products may exist. They mentioned that there was a discrepancy in the hydrogen balance too.

Some of the investigated strains in the group Chlorella vulgaris f. tertia, when analyzed for ethanol enzymatically, showed a positive result. But gas chromatographic analysis did not show the peak for ethanol as it has been shown for Chlorella fusca var. vacuolata. There was a peak which might be butanol. Confirmatory tests did not turn out to give positive results, but this may be due to different conditions when these experiments were conducted. Tests for 2,3-butanediol have not been carried out.

In identifying formic acid by different methods some difficulties had to be overcome. As far as identification by thin-layer chromatography is concerned, reproduction of the results varied. This might be due to the reasons given by the authors of the method (White and Starr, 1971). They mentioned that in some cases, for reasons that could perhaps be attributed to humidity or the condition of the adsorbent layer, the standard mixture did not separate adequately.

In addition to hydrogenase, lactic dehydrogenase is one of those enzymes which can be activated in algae under anaerobic conditions. The results shown in table 1 and 2 demonstrate that lactic acid in many of the Chlorella strains is produced as one of the main fermentative products of glucose. In spite of the results of Warburg et al. (1957) showing the configuration of lactic acid produced by Chlorella to be D(-) lactic acid, attention was not given to its importance for a long period. Recently Frederick et al. (1973) and Gruber et al. (1974) started investigating the configuration of lactic acid as related to the enzymes producing it. Gruber et al. (1974) concluded that in those green algae and other lower land plants where an enzyme produces D-lactate, it is related to glycolate dehydrogenase, and where the enzyme produces L-lactate it is related to glycolate oxidase. Frederick et al. (1973) showed the importance of the enzymes glycolate dehydrogenase and glycolate oxidase in determining the relationship between green algae and higher plants, concluding that species contain one or the other enzyme as a result of their development along separate evolutionary lines.

In the present investigation all those strains which produced lactic acid, when examined with D(-) lactic dehydrogenase, reacted positively indicating that it is D(-) lactic acid.

It may be reasonable to delineate the Chlorella species based on the production and non-production of lactic acid into separate groups. Once the presence of lactic dehydrogenase is firmly established in these strains, the enzyme lactic dehydrogenase, in addition to hydrogenase, can be utilized in grouping the Chlorella species based on the presence or absence of it.

Like enzymes of the type hydrogenase and lactic dehydrogenase, which become activated under anaerobic conditions, there are also other interesting and related enzymes such as alcohol dehydrogenase and formic dehydrogenase which can also be activated under anaerobic conditions. Among the Chlorella species studied, Chlorella fusca var. vacuolata showed a very active production of ethanol, which naturally may be due to the activity of the enzyme alcohol-dehydrogenase.

Although glyceric acid and related compounds received much attention in the studies of photosynthesis, glycerol as a fermentative product under anaerobic conditions was not paid attention to.

Kessler (1974) has demonstrated that the strains of Chlorella luteoviridis are able to tolerate 5% NaCl in their growth medium. The capacity to tolerate such high salt concentrations is remarkable for algae which have been isolated from fresh water. This makes it desirable to understand their behaviour under ecological stress or adverse conditions. The same group of Chlorella luteoviridis showed glycerol as one of the main fermentative products under anaerobic conditions. The other species producing glycerol under the same conditions were Chlorella saccharophila and Chlorella protothecoides. It is interesting to note that these species, too, exhibit a striking degree of salt tolerance (Kessler, 1974). Some strains of other taxa showed glycerol in traces only. Recent reports have hinted that there is some relationship between salt tolerance and an increased glycerol concentration in plant cells (Heimer, 1973).

Acetic acid and ethanol have been shown as fermentative products of glucose in Chlorella fusca var. vacuolata. Syrett and Wong (1963) also showed acetic acid as an important fermentative product in one of the strains of Chlorella fusca var. vacuolata.

The present work indicated that several Chlorella strains did not ferment glucose at all or only very slowly. Glucose estimation done after the experiments showed that much of it remained unutilized. However, these groups did form fermentative products which were liberated into the medium. It seems reasonable to assume that these substances were products of endogenous fermentation. Thus, Chlorella protothecoides produced lactic acid. Other species to be mentioned in this connexion are Chlorella luteoviridis, Chlorella kessleri and Chlorella saccharophila (except for the anomalous strains 211-1a and C-1.3.1).

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ZUSAMMENFASSUNG

87 Stämme der Gattung Chlorella wurden im Dunkeln auf die Produkte ihrer Glucose-Gärung untersucht. Die folgenden Produkte wurden identifiziert: Milchsäure, Essigsäure, Ameisensäure, Glycerin, Äthanol, Isopropanol, CO_2 und Wasserstoff. Die enzymatische Analyse zeigte, daß die Milchsäure erzeugenden Arten ausschließlich D(-) Milchsäure produzieren. Quantitative Untersuchungen ergaben, daß bei verschiedenen Arten unterschiedliche Mengen von Milchsäure pro Mol vergorener Glucose gebildet werden. Die stark salzresistenten Arten Chlorella luteoviridis, Chlorella saccharophila und Chlorella protothecoides produzieren erhebliche Mengen Glycerin. Die Gärungsprodukte können somit als weiteres Merkmal zur biochemischen Charakterisierung von Chlorella-Arten verwendet werden.

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